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HYPERPLASTIC NODULES IN RAT MAMMARY GLANDS FOLLOWING THE FEEDING OF 4-ACETYLAMINOBIPHENYL[1]

BY JULES TUBA, HERBERT E. RAWLINSON, M. SHIRLEY FRASER, AND IRMGAARD JESKE

Abstract

Virgin female rats did not respond to dietary 4-acetylaminobiphenyl by the production of tumors in mammary glands. This is attributed to some anti-carcinogenic factor(s) in the stock laboratory diet of Purina Fox Checkers, which are composed of natural foodstuffs. However, the carcinogen did stimulate gland development, and after eight months, hyperplastic nodules appeared in almost all the experimental animals but not in the controls. This indicates that suspected carcinogens may be having an effect on mammary tissue even though visible tumors may not be present.

Introduction

The study of chemical carcinogens has progressed to the stage where it is possible to feed certain compounds to experimental animals and to induce tumors in specific tissues. Sandin, Melby, Hay, Jones, Miller, and Miller (8) have synthesized compounds of this nature. One of these, 4-acetylaminobiphenyl, has been shown to produce tumors in mammary glands and ear ducts of female rats which had been maintained for eight months on a diet to which the carcinogen had been added.

Our studies on the oxygen uptake of rat mammary tissue (9, 10) led us to a consideration of the effect of 4-acetylaminobiphenyl on the cytochrome system during the period of tumor induction. At the same time changes in the histologically visible iron were followed by means of the staining technique described by Rawlinson (7). In this paper we report the histological findings with virgin female rats, and we have left the enzyme studies for a subsequent report.

[1] *Manuscript received October 6, 1952.*

Contribution from the Department of Biochemistry and the Department of Anatomy, University of Alberta, Edmonton, Alta. The investigation was supported by grants from the National Cancer Institute of Canada.

Experimental

Adult female virgin rats of the Wistar strain, three months of age, were housed in groups of 12 in large cages. They were given food and water ad libitum. Control animals were maintained on a diet of powdered Purina Fox Checkers, while the experimental animals received powdered Checkers, to which 4-acetylaminobiphenyl had been added to a concentration of 0.05%.

At various intervals during the experiment, indicated in Table I, both control rats and animals which were receiving the carcinogen were killed by decapitation. The left inguinal gland was removed at once from each animal and used for determination of the oxygen uptake. The hide was then removed along with the remaining mammary glands and fixed in 10% neutral buffered formalin (Lillie (3)) for at least 12 hr. Then the glands were removed from the skin; iron contamination was avoided as far as possible. These glands were stained for iron as outlined by Rawlinson (7), using potassium ferrocyanide and hydrochloric acid (2). They were cleared through alcohols and oil of origanum and examined in xylol. The iron content showed up the glands so that the connective tissue and fat could be dissected off under a binocular microscope and the glands examined quickly and thoroughly. Selected specimens were compressed between two pieces of glass held together by a small paper clip and immersed in xylol for a few hours when they were flat enough to mount in clarite or piccolyte under an ordinary cover glass for permanent mounts.

Results

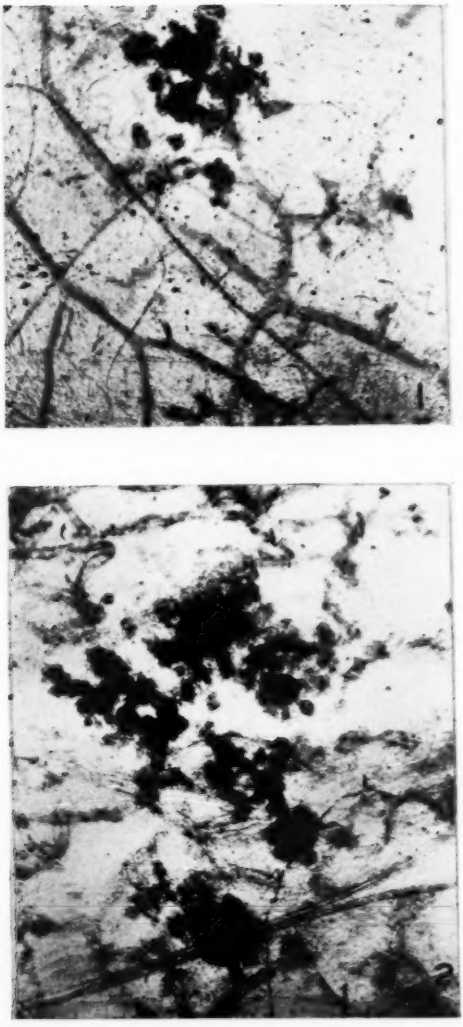
Instead of the usual eight month period used by Sandin *et al.* (8) for the induction of tumors by feeding carcinogen, it was found necessary to continue our experiment for 15 months. During this time observations were made on 36 animals which received the carcinogen-containing diet. One animal, after

TABLE I

THE EFFECT OF DIETARY 4-ACETYLAMINOBIPHENYL ON IRON DEPOSITION AND DEVELOPMENT OF RAT MAMMARY GLAND

No. months carcinogen was eaten	No. of animals	Histological changes in right inguinal gland and fore glands
4	1	Very little iron. Poor development
5	6	Ditto
6	6	One animal had two small hyperplastic nodules in inguinal gland
8	6	Hyperplasia and small nodules in four animals. All glands showed good development and heavy iron staining
11	6	Hyperplastic nodules in glands of five animals
13	5	Four animals had hyperplastic nodules
15	6	Four animals had hyperplastic nodules

PLATE I



FIGS. 1 AND 2. Section from mammary gland of virgin female rat fed carcinogenic diet for 15 months. Stained for iron by the use of hydrochloric acid and potassium ferrocyanide, ducts of the gland show up faintly, but hyperplastic nodules are very prominent and appear black because of the stained iron. Magnification ×20, whole-mount of the gland.

13 months on the diet, had a swollen area in one mammary gland, which on section appeared to be a fibro-adenoma. Another animal, also after 13 months, developed an abscess in the ear. Three rats developed tumors on the bladder after 12–15 months on the experiment. These were not studied histologically. Gross extravasation of blood into the uterine horn was noted in five animals in the 13–15 month group. The majority of the rats which were fed 4-acetylaminobiphenyl had very large spleens.

The histological findings, which are summarized in Table I, indicated that hyperplastic nodules began to appear in the mammary tissues after six months on the diet. In the experimental period from 8–15 months, 17 out of 23 animals had developed these nodules, as well as irregular areas of hyperplasia.

In 20 control animals, there was limited gland development and iron deposition, and in no case were any nodules found. These rats, which were killed two or three at a time, at the intervals indicated in the table, were always the same age as the corresponding experimental animals. It thus appears that after eight months of a carcinogenic dietary regimen, hyperplastic nodules are the rule, with few exceptions.

It is well known that hyperplastic nodules occur frequently in strains of mice with a high incidence of spontaneous mammary cancer. Rawlinson (7) has demonstrated that such hyperplastic nodules can accumulate iron and that they show up well in the whole mounts stained for iron. Typical hyperplastic nodules produced by carcinogen feeding are shown in Figs. 1 and 2.

Discussion

Sandin *et al.* (8) report that out of 15 animals fed 4-acetylaminobiphenyl with a grain diet for eight months, 11 developed tumors in the mammary gland and two in the ear duct. The fact that we found two small tumors only in 36 animals during a 15 month feeding period may be attributed to unknown substance(s) in our stock laboratory diet of Purina Fox Checkers. It is well known that a number of substances that are characteristic of natural diets can affect the outcome of feeding certain carcinogens and Miller (5) has reported on dietary factors which decrease the carcinogenicity of azo dyes. Miller, Miller, Sandin, and Brown (4) have found that a change from a semisynthetic to a grain diet resulted in a greater degree of carcinogenicity with 2-acetylaminofluorene. The Wisconsin workers have also found that at least one commercial chow greatly delays the production of tumors by 2-acetylaminofluorene (6). Engel and Copeland (1) have proved that semipurified or purified diets are preferable to stock diets of natural foodstuffs for production of tumors in young female rats with 2-acetylaminofluorene.

It would appear that with our diet and under the conditions described the carcinogen was unable to produce definite tumors, but the stimulus was sufficient to result in the production of hyperplastic nodules. The experiment is at the present time being repeated with a purified diet, in the hope that actual tumor production will occur.

Our findings indicate the possibility that potential carcinogens may cause changes in the mammary glands of rats even in the absence of definite tumors. It must be emphasized that the relative absence of tumor formation reported here, however, concerns virgin animals only. Experiments under way indicate that older rats that have been used as breeders react more readily to 4-acetylaminobiphenyl by the formation of tumors.

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SEX DIFFERENCE IN RESISTANCE OF RATS TO THYROID HORMONE[1]

By R. D. Prueter, M. D. Warson, and J. K. W. Ferguson

Abstract

Two series of experiments were performed to compare the metabolic responses of male and female rats to thyroid hormone. In the first series dried thyroid was fed in the diet at a concentration of 1.5 gm. per kgm. of chow. Initial body weights ranged from 130 to 160 gm. Oxygen consumption was measured with the rats lightly anaesthetized with pentobarbitone sodium. In the second series, sodium thyroxine was injected subcutaneously once a day for five days and oxygen consumption was measured without anaesthetic. Initial body weights averaged 147 gm. for males and 137 gm. for females. Two doses of sodium thyroxine were used, 0.1 and 0.5 mgm. per kgm. per day. In both series the increase in oxygen consumption following the administration of thyroid or thyroxine was much less for female rats than for the males. In Series I, half the animals were castrated. The castrated females responded to thyroid in the same way as the normal and castrated males. The castrated females were then given stilboestrol (5 mgm. per liter in drinking water) for three weeks while they were kept on the same diet containing thyroid. Their metabolic rate returned to the levels of unoperated females. Light anaesthesia during the measurement of oxygen consumption reduced the variance within groups with high rates of oxygen consumption but not in groups with normal rates.

In the course of measurements of oxygen consumption in rats on a diet containing dried thyroid, it was observed by one of us (R.D.P.) that the response of female rats was apparently much less than that of male rats. A search of the literature revealed that Schoeller and Gehrke (4) had reported that injection of thyroxine into mice caused a smaller rise in carbon dioxide production in females than in males. Since we were unable to find confirmation of these observations in mice or in other species, the following experiments were undertaken.

Methods

Oxygen consumption was measured by a closed circuit volumetric technique using a battery of four transparent plastic chambers immersed in a water bath at 30° C. Each chamber contained one rat and a quantity of Baralyme to absorb carbon dioxide. The chambers were flushed with oxygen for 15 min. after which each chamber was connected to a graduated cylinder, which in turn was connected by a narrow tube open to the water in the bath. As oxygen was used, the pressure in the chamber and the graduate fell and water from the bath entered the graduate. Oxygen consumption was measured for a period of 15 or 20 min. by the volume of water entering the graduate. From the oxygen consumption per minute and the weight of the rat, the metabolic rate was computed in cubic centimeters of oxygen (N.T.P.) per square meter of body surface per minute, using a nomogram.

Two series of experiments were performed. In Series I, dried thyroid (Canadian standard containing 0.3% iodine) was fed in powdered fox chow in a concentration of 1.5 gm. per kgm. of chow. Initial body weights ranged

[1] *Manuscript received November 27, 1952.*
Contribution from the Department of Pharmacology, University of Toronto, Toronto, Ont. Aided by a grant from the Banting Research Foundation.

from 130 to 160 gm. Oxygen consumption was measured with the rats lightly anaesthetized with pentobarbitone sodium given intraperitoneally in a dose of 1 mgm. per 100 gm. body weight. In Series II, sodium thyroxine was injected subcutaneously each day for five days. Two doses were used, 0.1 and 0.5 mgm. per kgm. Oxygen consumption was measured without any sedative.

Series I and II differed also in duration and design of the experiments. In Series I, 64 rats were used, 32 males and 32 females. Half the males and half the females were castrated and given three weeks to recover from the operation. Since 64 animals could not be handled concurrently, the experiment w s done with eight sets of eight rats started a week apart. Each set contained two normal and two castrated males and two normal and two castrated females. Oxygen consumption was measured without preliminary starvation, before and after a period of three weeks during which the animals ate the diet containing dried thyroid. For each pair of castrated female rats the experiment was prolonged for another three weeks during which they were given stilboestrol in drinking water (5 mgm. per liter in 0.01 *M* sodium hydroxide) while they were kept on the thyroid diet. After three weeks, oxygen consumption was measured again.

In Series II, 32 males and 32 females were used. Average initial body weights (± S.D.) were: 147 ± 15 gm. for males and 137 ± 9 gm. for females. None were castrated. Oxygen consumption was measured at the same time each day for five days without preliminary starvation. After the first measurement of oxygen consumption the animals were injected subcutaneously with thyroxine sodium and reinjected daily thereafter for four more days.

Observations

The results of Series I are summarized in Table I. The metabolic rate of females was slightly lower than that of males; that of castrates was slightly lower than that of noncastrates. After the feeding of dried thyroid the metabolic rate of the unoperated females did not increase significantly; that of the other three groups increased by 30% or more. When the castrate females were given stilboestrol their metabolic rate returned to normal, although they were kept on the same diet containing dried thyroid.

TABLE I

AVERAGE OXYGEN CONSUMPTION FOR GROUPS OF 16 RATS

(In cc. per 100 $cm.^2$ per min.)

	Males		Females	
	Unoperated	Castrate	Unoperated	Castrate
Before thyroid	123	119	118	116
After thyroid	153	152	122	157

NOTE: *S.E. of means of groups of 16 = ± 3.0.*
Differences between means of the groups are significant.
($P < 0.05$) if larger than 10.

TABLE II

AVERAGE BODY WEIGHTS (± S.D.) IN GRAMS, FOR GROUPS OF 16 RATS, SERIES I

	Males		Females	
	Unoperated	Castrated	Unoperated	Castrated
Before thyroid	176 (± 13)	174 (± 16)	171 (± 17)	172 (± 13)
After thyroid	211 (± 18)	209 (± 21)	204 (± 15)	201 (± 15)
After stilboestrol and thyroid	—	—	—	224 (± 13)

In Table II the average body weights of the four experimental groups show that the dose of thyroid was not such as to interfere with normal growth in these animals.

The results of Series II are displayed in Fig. 1. In these experiments the increase in metabolic rate was greater, which may be due in part to the omission of the anaesthetic. Both males and females responded to the injections of sodium thyroxine with a rise in metabolic rate. The increase in males is significantly greater than in females ($P < .01$ at the higher dosage).

In spite of the omission of anaesthetic the variance of oxygen consumption within groups of untreated animals was no greater than in Series I. However, as the metabolic rate increased under the influence of thyroxine, the variance increased in Series II but not in Series I. At the higher metabolic levels, the variance within groups was significantly greater in Series II than in Series I ($P < 0.02$). It seems that under the influence of thyroxine the restlessness of the unanaesthetized animals contributes significantly to individual variations.

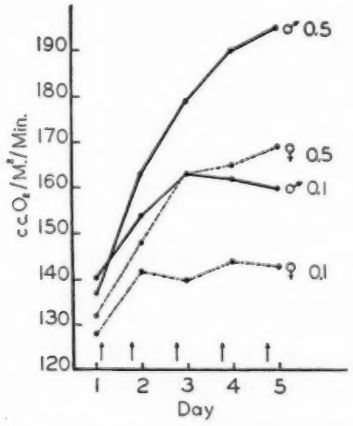

FIG. 1. Oxygen consumption of male and female rats injected daily with sodium thyroxine, 0.1 or 0.5 mgm. per kgm. body weight.

Discussion

The results of Series I alone might have been ascribed to a difference between males and females in the absorption of thyroid hormone from the alimentary tract, or perhaps to a greater effect of the anaesthetic (pentobarbitone sodium) in depressing metabolism in the females receiving thyroid. Series II demonstrates that the sex difference cannot be ascribed to these factors. A more direct kind of antagonism must be postulated between thyroid hormone and ovarian hormones or stilboestrol, in the sense that the latter may reduce the response of tissues to thyroxine or increase the inactivation or elimination of thyroxine. Our results with stilboestrol are consistent with observations by Gessler (2) who reported that 0.1 mgm. of oestradiol injected daily into guinea pigs antagonized the effect of dried thyroid given orally. His results were obtained on one male and five female guinea pigs. The antagonism of ovarian and thyroid hormones may be described as mutual, since several authors have reported that thyroid hormones decrease the response of female rats to administered oestrogenic hormone (1, 3, 5).

All authorities agree that thyroid disease occurs much more frequently among women than among men. This is usually attributed to greater activity of the thyroid gland in women as a result of menstrual and reproductive functions. Data which we are now preparing for publication indicate that the rate of production of thyroid hormone is greater in female rats than in males. It is not possible to say yet how this increased activity is brought about. The demonstrable antagonism between stilboestrol and thyroxine offers a clue which merits further investigation.

Two additional points of interest for the design of experiments in this field were revealed in our data. (1) Individual variations in oxygen consumption were no greater among the female rats than among the males, either in untreated animals or those treated with thyroid or thyroxine. (2) Conversion of the figures for oxygen consumption of individuals to oxygen consumption per unit of body surface did not diminish the coefficient of variation in any experimental group, even though the animals in a group varied considerably in body weight. The range of body weights in each group was usually 30–40 gm. The computation of oxygen consumption per unit of body surface appeared to serve one useful, or at any rate plausible, purpose. It seemed to provide a suitable adjustment for the growth of rats during the three weeks occupied by the experiments.

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MITOCHONDRIAL POPULATION OF MAMMALIAN CELLS

III. NUMBER OF MITOCHONDRIA PER AVERAGE CELL OF RAT LIVER TUMOR INDUCED BY 4-DIMETHYLAMINOAZOBENZENE. SIGNIFICANCE IN THE COMPARATIVE STUDY OF THE MITOCHONDRIAL FRACTION PROPERTIES OF NORMAL TISSUES AND TUMORS[1]

By Claude Allard, Gaston de Lamirande, and Antonio Cantero

Abstract

The number of cells per gram of fresh tissue and the number of mitochondria isolated from rat liver and liver tumor homogenates are different. The mitochondrial population, computed from the number of cells and mitochondria per gram of fresh tissue is effectively decreased to about half the normal level in 4-dimethylaminoazobenzene induced hepatic tumor cells. Such a loss of mitochondria in tumor cells might explain some different biochemical properties of the mitochondrial fraction isolated from hepatic tumor cells. Fundamentally a decrease in the mitochondrial population favors a recent hypothesis of carcinogenesis.

Introduction

Many fundamental enzymatic processes and important chemical constituents are specific to, or found in, the mitochondrial fraction isolated by centrifugation from broken mammalian and plant cells (6, 18). Some mitochondrial properties appear different in tumors and in precancerous conditions as compared to normal tissue (9, 14, 18). In the course of a study of the distribution of biochemical properties in the rat liver cell particulates during general damage (2), it appeared essential to estimate mitochondrial properties in terms of the average mitochondrion. A quantitative method of counting mitochondria in rat liver homogenate and in cellular particulates obtained by centrifugation in $0.25M$ or $0.88M$ sucrose has been devised in this laboratory (5). When the number of cells is known in conjunction with the number of mitochondria per gram of fresh tissue the mitochrondial population of the average cell may be computed (5). It seems possible that variation in the number of mitochondria per cell in different physiological and pathological conditions might reveal an hitherto unknown general mechanism to injury and possibly to carcinogens. Previous experiments have shown that, in normal stimulated growth of the liver occurring after partial hepatectomy, the average cell contains fewer mitochondria (3). The present investigation is concerned with the number of mitochondria isolated from pure rat liver tumor induced by 4-dimethylaminoazobenzene (DAB). The average mitochondrial population of the liver tumor cell is computed.

[1] *Manuscript received April 28, 1952, and, as revised, August 13, 1952.*

Contribution from the Montreal Cancer Institute, Research Laboratories, Notre-Dame Hospital, Montreal, Que. This investigation is being supported by a grant from the National Cancer Institute of Canada.

Material and Methods

The liver tumors used in this study are induced by feeding adult Wistar rats a low-protein semisynthetic diet (11, diet 3) to which 0.06% of DAB is incorporated. After 150 days of continuous feeding, the animals are put back on a complete diet (Purina Fox Chow) with water ad libitum, for two months. As far as possible, only nonnecrotized tumors of about 1 cm. diameter, carefully excised from liver, are used for quantitative determinations of the number of mitrochondria.

The preparation of tissue homogenate in 0.25 *M* sucrose and the subsequent centrifugal separation in three cellular components, namely, a nuclear (N), a mitochondrial (M), and a supernatant (S) fraction, are exactly the same as described previously (5) and correspond essentially to a separation scheme elaborated by Schneider and Hogeboom (17). The number of mitochondria present in the separate isolated fractions are estimated by a method described in a previous publication (5). The cell counts are done according to the Price and Laird technique (13). Total nitrogen content (TNC) is determined by micro-Kjeldhal procedure.

Results

Table I shows the mean total number of mitochondria and cells per gram of fresh tissue, the percentage of mitochondria present in the (N) fraction, and the mean number of mitochondria per cell. The confidence limits for

TABLE I

MEAN NUMBER OF MITOCHONDRIA PER AVERAGE CELL OF RAT LIVER, LIVER TUMOR, AND LIVER FREED FROM TUMORS; AND RAT LIVER WITH TUMORS DURING FEEDING OF DAB-SEMISYNTHETIC DIET

Tissue	Mean number of mitochondria $\times 10^{-10}$ per gram-equivalent of fresh tissue	Percentage of mitochondria present in the (N) fraction	Mean number of cells $\times 10^{-6}$ per gram of fresh tissue	Mean number of mitochondria per average cell†
Liver	33.0†† ± 1.1 (11)	10.6	129.2 ± 4.4 (42)	2554
Liver tumor	12.3 ± 5.2 (4)	26.4	88.4 ±20.0 (8)	1391
Liver freed from tumors	22.8 ± 2.8 (3)	22.4	121.3 ± 5.7 (6)	1880
Liver with tumors†††	30.4 ±13.1 (5)	—	99.6 ± 7.5 (12)	3052

† *Assuming mononuclear cells.*
†† *Previously published (6).*
††† *See text.*

the mean, with a probability of 0.95, and the number of animals are respectively indicated beneath the mean and in parentheses. The (S) fraction is not included since this fraction contains no detectable mitochondria. Table I reveals that, on a per gm. of fresh tissue basis, the mean number of mitochondria is decreased about three times in tumor tissue and one and one-half times in the liver freed from tumors. There is also a striking decrease in the number of cells per gram of fresh tumor and liver freed from tumors. The difference with normal value is statistically significant with a probability of 0.95.

The mean number of mitochondria of the average liver tumor cell is 1391. The probable range of the mitochondrial population is 1038 to 1614. The range is calculated with the respective confidence limits of the means of the number of mitochondria and cells (Table I). The mitochondrial population of the liver freed from tumor cells is 1880 which falls within a range of 1730 to 2000.

Preliminary results concerning the rat liver tumor mitochondrial population (5) do not seem to be in accordance with the above findings. Values higher than normal were observed. The discrepancy seems to be due to the fact that the animals received the carcinogenic diet continuously until sacrificed and that relatively large tumors were employed. Table I also shows results obtained with total liver bearing large tumors taken from rats fed the carcinogenic diet continuously for about 200 days. The population of the average cell of tumor-bearing liver, in such dietary and experimental conditions, is higher than normal. The phenomenon is presently under investigation.

As previously observed (5), the mitochondria present in the *mitochondrial* fraction isolated from tumor cells seem smaller than normal mitochondria. It may be worth mentioning also, that much larger mitochondria than normal seem to be present in the *nuclear* fraction. A scheme for the separation of these physically different mitochondria and the determination of their relative number is presently being examined.[1]

That the low cell and mitochondrial counts in liver tumor are not due to rupture of these cell components during the process of homogenization has been confirmed. Table II shows the number of cells and mitochondria per gram of fresh tumor tissue after different intervals of time of homogenization. As a general rule, between two and one-half and three minutes of homogenization was used and found to be sufficient to obtain maximum cell rupture.

Table III shows the mean corrected[2] TNC of the mitochondrial fraction isolated from liver, tumor, and liver freed from tumors. TNC is given on a fresh weight and on a mitochondrion basis. Previously published data (3) for regenerating liver, during the first day after partial hepatectomy, and for immature rats are also included in this latter table. It illustrates that the

[1] *Since the present paper was sent for publication, Novikoff et al. published a method for the separation of rat liver mitochondria of different size and biochemical properties. (J. Histochem. Cytochem. 1 : 27. 1953.)*

[2] *Corrected for the number of mitochondria present in the (N) fraction (5).*

TABLE II

EFFECT OF DIFFERENT INTERVALS OF TIME OF HOMOGENIZATION ON THE QUANTITATIVE DETERMINATION OF THE NUMBER OF MITOCHONDRIA AND NUCLEI PER GRAM OR AN EQUIVALENT OF FRESH LIVER TUMOR

Interval of time of homogenization† (minutes)	Nuclear count		Mitochondrial count	
	A	B	A	B
2½	106	65	16	8
5	98	61	18	7
15	76	58	19	8

† *With glass Potter–Elvehjem homogenizer. Pestle turns at 300 r.p.m.*

TABLE III

MEAN CORRECTED TOTAL NITROGEN CONTENT (TNC) OF THE MITOCHONDRIAL FRACTION AND OF THE AVERAGE MITOCHONDRION

Tissue	Mgm. TNC of (M) fraction per gram-equivalent of fresh tissue	Gram TNC $\times 10^{-15}$ per mitochondrion
Normal rat liver	6.60 (11)†	22.3
Pure rat liver tumor	3.81 (4)	30.8
Liver freed from tumors	6.52 (2)	28.6
Liver after one day of partial hepatectomy	6.76 (5)	28.6
Immature rat liver	7.28 (2 pools of 4 and 3 rats)	28.5

† *Number of animals.*

average liver mitochondrion seem to have a higher TNC in these different physiological and pathological conditions. It is interesting to mention that the percentage composition of soluble protein of the mitochondrial fraction has been found similar in various conditions of liver damage where the mitochondrial population is about 65% of the normal level (8).

Discussion

The estimation of the liver cell mitochondrial population is based on two separate experimental procedures. A method of counting mitochondria and a method of counting cells (nuclei). The accuracy of these techniques has been established (5, 13). It has been shown that the direct counting of mitochondria in liver homogenate, though possible, is more difficult. When dealing with liver tumor homogenates the direct counting of mitochondria seems of doubtful accuracy because mitochondria cannot be recognized as easily as in the cellular fractions. For this reason and also because the number of mitochondria isolated in the mitochondrial fractions was needed for other studies these were determined in the separate fractions isolated by centrifugation.

The fact that 1 gm. of fresh tumor pulp[3] contains about 88 cells as compared to 129 cells per gm. of liver is bewildering. This seems incompatible with the known fact that tumor cells are much smaller than liver cells (15). Cell volume measurements made in this laboratory show that the average tumor cell is about three times smaller than liver cell. In fact, the mean diameter of normal rat liver nucleus is found to be 8.02μ in accordance with recent data (1). Assuming a spherical nucleus, the volume is $270\mu^3$. The liver average cell diameter is about 21μ which gives a volume of $163\mu^3$ assuming the liver cell to be a regular octahedron. The cytoplasmic–nuclear volume ratio is thus 5.05 which is confirmatory (15). The average volume of DAB-induced tumor nucleus is $145.9\mu^3$. With a cytoplasmic–nuclear ratio of 3.04 (15), the tumor cells have a calculated volume of $589\mu^3$ which is approximately three times smaller than liver cells.

Consequently, on a volume basis, the number of cells in liver tumor is probably about three times more numerous than in normal liver cells. However the cells are not counted on a volume basis but rather on a wet weight basis of tumor pulp. The low cell counts of tumor might be due to an increased weight of the tumor cells and also to the fact that when fresh tumor tissue is squeezed to prepare the pulp prior to homogenization and counting, more extracellular matter, such as water, is weighed. In fact, a high water content is characteristic of neoplastic tissue (10). This problem requires investigation.

The number of mitochondria per average cell of tumor and of liver freed from tumors has been shown to be lower than in healthy liver cells. A decrease of the same amplitude has also been observed in regenerating liver after partial hepatectomy (3) and in liver of rats fed DAB and other carcinogens for periods insufficient to produce tumors (4).

These results appear to support an hypothesis of carcinogenesis elaborated by Potter *et al.* (12). The fact that liver freed from tumors, precancerous liver, and regenerating liver have a decreased mitochondrial population comparable with that of tumor, favors the opinion that an irreversible change occurs in the average cell prior to the appearance of tumor (12). Speculating further, it is not impossible that conditions (diet and irritation) inducing a loss of mitochondria in the cell might enhance a tendency of the cell to multiply, and that such a comportment might become hereditary owing to the continuous action of a carcinogen over a certain period of time. To an irreversible change would respond the appearance of cancer cells.

Some hereditary abnormalities in cells of certain plants have been shown to be due to mutant mitochondria (9, 20). Furthermore these mutant mitochondria might be the "continuing cause of plant neoplasia characterized by distinctive changes in the cellular enzymatic activities" (9, 19). Presently, as expressed by others (20), there is no evidence that plant mitochondria are different from animal mitochondria. The search for any abnormalities in the latter group thus becomes quite interesting. However investigation of

[3] *Prepared by forcing the tumor tissue through a plastic squeezer prior to homogenization.*

morphological abnormalities in mammalian mitochondria (7) is greatly hindered by technical difficulties. As for *biochemical* abnormalities, it has seemed rather difficult to ascertain whether these were due to defective mitochondria or to variation in the number of these units in the cells. The use of the mitochondrion as a unit will facilitate the interpretation of future results.

For example it is known that important enzymatic processes of the mitochondrial fraction are deleted to some extent in tumor as compared to normal liver (18). Since the number of mitochondria per gram of fresh DAB-tumor appear two to five times less than in liver (Table I) it seems reasonable to state that biochemical properties of the mitochondrial fraction found two to five times less (on a fresh weight basis) in tumor, might probably be attributed to a decrease in their number.

It has been shown in this laboratory that adenosinetriphosphatase and desoxyribonuclease activity, on a mitochondrion basis, is about the same in liver as in tumor[4], though the activity, on a fresh weight basis, is about two to four times less in the tumor mitochondrial fraction (16). Obviously, all the mitochondrial properties, known to be different in tumor cells, cannot be explained as in the case of ATP-ase or DN-ase. However, defects may be attributed to the mitochondrion if this cellular particle is used as a unit of measurement.

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[4] *Unpublished data.*

FURTHER STUDIES ON THE NEUROLYTIC PROPERTIES OF THE SPINAL FLUID IN HEALTH AND DISEASE[1]

By Ernest Kovacs

Abstract

The "neurolytic" property of the cerebrospinal fluid of neurologically normal and of various clinical cases was examined with buffered bovine brain-cord emulsion (brei) as substrate. The splitting of the inactivated nerve tissue emulsion was determined by measuring the changes of the acid soluble inorganic phosphorus and of reducing substances, expressed as sugar, before and after incubation. From our results three main groups may be distinguished: specimens of normal or neurologically normal individuals with negative laboratory findings in the cerebrospinal fluid, without neurolytic activity; specimens from patients with inflammatory processes in the central nervous system, with corresponding pathological changes in the cerebrospinal fluid and parallel high neurolytic activity; spinal fluids from degenerative and proliferative processes with moderate changes in the cerebrospinal fluid, exhibiting high neurolytic activity. The significance of neurolysis and the possible source of the enzymes are discussed.

Introduction

The earliest contribution to the literature of the neurolytic properties of cerebrospinal fluid (CSF) was that of Speransky (16), who showed that by freezing the brain cortex of dogs the CSF of such animals had a marked damaging effect on brain tissue *in vitro* and also when introduced subdurally, *in vivo*. He concluded that the observed changes were due to the action of "neurotoxins". By histological technique Bruschettini (1), Meco (15), and Greco (7) proved that when nerve tissue is incubated in CSF it undergoes certain changes which they termed neurolysis. Greco (7) concluded that the neurolysis was due to the action of enzymes. When the CSF was heated to 60–70° C. its neurolytic property disappeared. Agitation and changes of temperature had a marked effect on the process. Normal CSF according to Greco has no neurolytic action. He maintained that this process is checked normally *in vivo* either by the intact circulation or by enzyme inhibitors. In his assays buffers were not used and physiological saline served for the incubation of controls. He claimed that, under these conditions, neurolytic changes were not found. He and all the previous workers used various time intervals for incubation at 40° C. Kovacs (11, 12) proved by biochemical means that the neurolysis was enzymatic in character. He used well-buffered systems of brain tissue emulsion and CSF and measured the changes of inorganic phosphorus and of carbohydrate content under sterile conditions before and after incubation at 37° C. Different patterns for normal and for pathological cases were found.

In the present study results on a greater number of specimens of CSF using improved technical methods and using enzyme activators and inhibitors are reported.

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Contribution from the Department of Hygiene and Preventive Medicine, University of Toronto, Toronto, Ont. This work was financed by the Hastings Memorial Fellowship of the University of Toronto and in part by a Mental Hygiene grant.

Methods

Substrate

As substrate a 5% emulsion of bovine brain and cord tissue (BCE) of equal proportion in physiological saline was used. The fresh tissue was minced and extracted with acetone to remove water; the acetone was evaporated under vacuum and the residue added to the dried tissue, which was emulsified in a Waring blendor. Inactivation of the nerve-tissue enzymes was carried out for two days starting at 45° C. and gradually increasing to a final temperature of 60° C.; sterility tests were carried out and 0.3% toluene was added.

Technique

Two milliliters of the CSF was pipetted to 8 ml. of 5% BCE in a glass-stoppered sterile bottle. In the phosphorus assays, 10 ml. of 0.2 *N* veronal buffer, pH 8.5, and in the assays for reducing substances, 10 ml. of 0.2 *M* phosphate buffer, pH 5.5, was added. The total volume of the system was thus 20 ml. Two drops of chloroform were added as preservative to each bottle. Frequent sterility tests on blood agar or broth have shown no infection, although such tests were not carried out for each specimen examined. Before and after incubation the acid-soluble inorganic phosphorus was assayed by the Fiske-Subbarow method as modified by King (10), and the reducing substances of the system by Benedict's method (6). The difference between the initial and end values was expressed as milligrams per cent of the system and indicated the degree of neurolysis. As a rule, the incubation was carried out at 37° C. for 24 hr., but the effect of lower and higher temperatures and different time intervals was investigated. Both fresh and heat inactivated CSF and substrate and reagent blanks served as controls throughout these assays and their values were deducted in the calculation of results. Magnesium sulphate or calcium chloride in 0.01% final concentration was used in the assays for activators and 2 ml. of *Clostridium welchii* antitoxin* in the inhibition experiments. The aliquots for determinations were always taken from the supernatant after centrifugation.

Results

Over 300 determinations were made on the CSF of 92 persons. In order to economize space only illustrative examples from these results are shown in tabular form. Table I, Group *A* shows examples from the results of the CSF assays on 15 normal or neurologically normal persons. It will be noted that the changes in acid soluble inorganic phosphorus concentrations are slight. No values higher than 2 mgm.% were obtained, including the experimental error. Though the number in this group of normals is small, one may be tempted to conclude that there is no neurolytic activity normally evident in these spinal fluids. This is in agreement with the findings of Greco (7) and others.

* *This was kindly supplied by the Connaught Medical Research Laboratories, Toronto, Ont.*

TABLE I

EFFECT OF CSF ON INORGANIC ACID SOLUBLE PHOSPHORUS CONTENT OF SUBSTRATE

Name	Diagnosis	Laboratory notes	Change in acid soluble inorganic phosphorus, mgm. %
Group *A*—Normal, neurologically normal persons (neg. laboratory findings in the CSF). No or slight neurolysis			
F.D.	Acute nasopharyngitis	No cells. Pa : neg.	1.0
B.C.	Hernia inguinalis	No cells Pa : neg.	0.1
A.M.	Sine morbo	No cells. Pa : neg.	0.5
B.W.	Influenza	No cells. Pa : neg.	0.2
G.D.	Influenza	No cells. Pa : neg.	1.7
E.M.	Gastritis	5 lycytes.* Pa : neg.	0.7
L.S.	Cured meningitis	L.P.** after 6 months, 10 lycytes. Protein 35 mgm. %	2.0
Group *B*—Acute inflammatory diseases (high cell count and proteins in the CSF). High neurolysis			
S.G.	Meningococcal meningitis (acute stage)	600 WBC. 90% Polys. Prot: 72 mgm. %	14.0
MC.K.P.	Influenzal meningitis	1200 WBC. 95% Polys. Prot: 95 mgm. %	8.0
L.R.	Pneumococcus meningitis	400 WBC. 80% Polys. Prot.: 58 mgm. %	4.0
S.M.	Strept. meningitis	800 WBC. 98% Polys. Prot: 120 mgm. %	6.3
S.S.	Tuberculous meningitis	70 WBC. 80% Polys. Prot: 60 mgm. %	12.1
C.R.	Polioencephalitis	40 lymphocytes. Prot: 52 mgm. %	10.0
B.N.	Lymphocytic meningitis	100 lymphocytes. Prot: 45 mgm. %	6.0
H.P.	Mumps encephalitis	32 cells. Pa : pos.	3.4
Group *C*—Mixed cases (low cell count and inflammatory reactions). High neurolysis			
L.L.	Subdural haematoma	Haemolyt. CSF. Pa : traces	3.3
S.H.L.	Fracture of skull, hernia cerebri	15 WBC. Pa : neg.	3.3
Cr.D.	Cerebral birth injury dystrophy	No cells. Pa : neg.	9.0
Fl.G.	Febrile convulsions (infant)	5 lycytes. Pa : neg.	10.0
F.Gr.	Hydroceph. meningocele	15 lycytes. Prot : 35 mgm. %	13.0
Z.N.	Sarcomatosis (generalized)	30 lycytes. Prot: 60 mgm. %	9.2
M.L.	Amyotrophic lat. sclerosis	No cells. Prot : 40 mgm. %	7.2
M.A.	Alcoh. encephalopathy	No cells. Prot : 60 mgm. %	6.3
M.N.	Paralyt. polio? (Guillain-Barré, post-vacc. enceph.?)	10 lycytes. Prot : 200 mgm. %	16.0
N.N.	Tabes dorsalis	Blood Wass. Re : pos. Koll: neg. CSF. Wass. Re: pos. Prot: 48 mgm. %	5.0
D.Mc.L.	Neurosyphilis	30 lycytes. Re : pos. Koll: neg. CSF. Wass. Re: pos. Prot. 42 mgm. %	4.0

* *Lymphocytes.* ** *Lumbar puncture.*

In contrast to the normals, Group *B* shows eight examples of 15 cases of acute and subacute inflammatory neurological conditions with the corresponding CSF findings. This group is characterized by marked neurolysis. Highest values were found in meningococcal and tuberculous meningitis. In Group *C* are presented 11 examples of a group of 20 miscellaneous cases. Most of these represent chronic neurological conditions. The grade of neurolysis ranges from significant to very high values. The highest value

TABLE II

EFFECT OF CSF ON REDUCING SUBSTANCE CHANGES OF SUBSTRATE

Name	Diagnosis	Laboratory notes	Changes of the reducing substance content of the system as glucose,* mgm. %
Group *D*—Acute inflammatory diseases (high cell counts and proteins in CSF). High neurolysis			
G.T.	Meningococcal meningitis	3500 cells. 90% Polys. Prot: 150 mgm. %	+26
B.W.	Influenzal meningitis	600 cells. 92% Polys. Prot: 80 mgm. %	−13
W.S.	Influenzal meningitis	800 cells. 95% Polys. Prot:	−60
F.D.	Strept. meningitis	1000 cells. 100% Polys. Prot: 100 mgm. %	−26
R.D.	*E. coli* meningitis	85,000 cells. Mostly Polys. Prot: 400 mgm. %	+74
W.C.	Tuberculous meningitis (under treatment)	30 WBC. Pa: pos.	−10
B.N.	Lymphocytic meningitis	100 lycytes. Pa: pos. Prot: 52 mgm. %	No change
Group *E*—Chronic inflammatory and degenerative processes (low cell count and protein in CSF). High neurolysis			
N.F.	Tabes dorsalis	Cells 10 lycytes. Prot: 60 mgm. %	+25
K.T.	Juvenile paresis	Ser: + Wass. CSF: + Wass. cells: 25. Prot. 52 mgm. %	+22
G.B.	Neurosyphilis	Blood Wass. Re:+. CSF Wass.: + No cells. Prot. 40 mgm. %	+68
T.B.	Febrile convulsions	No cells. Pa: neg.	+14
H.A.	Idiopathic epilepsy	No cells. Pa: neg.	+13
H.T.	Hemiplegia (vasc. origin)	No cells. Pa: pos.	+32
B.N.	Atrophy of the brain (dementia)	No cells. Pa: neg.	+95
M.L.	Amyotrophic lateral sclerosis	No cells. Pa: neg.	+24
H.N.	Cyst of brain	No cells. Pa: neg.	+25
S.H.	Concussion, hernia cerebri	No cells. Pa: neg.	+15
L.L.	Subdural haematoma	Haemolyt. CSF	−15

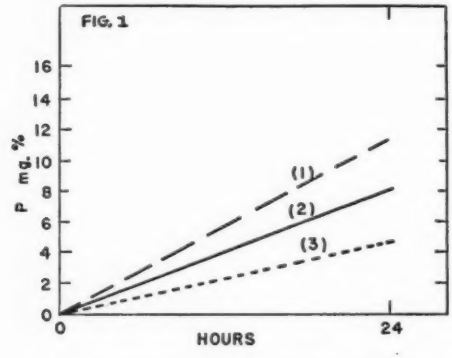
* + *Indicates increase of the value compared to the original.*
− *Indicates decrease.*

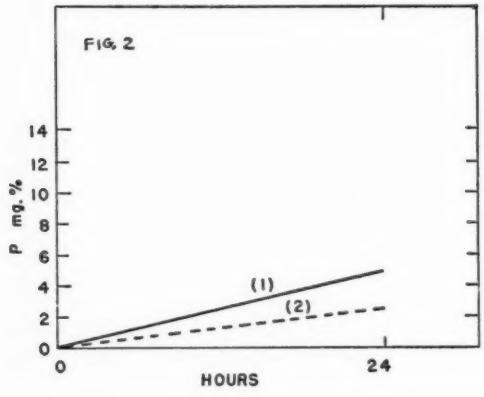
obtained was in a case of dubious etiology (paralytic poliomyelitis or Guillain-Barré syndrome or postvaccinal encephalitis). In this case proteins were high in the CSF in spite of the presence of only a few lymphocytes. Table II, Group *D* reflects the changes in reducing substances of the system in 8 examples chosen from 15 acute or subacute meningitis cases. The significance and interpretation of these findings will be discussed later. In Table II, Group *E*, characteristic samples are taken from 27 chronic and, in the main, degenerative conditions. In such neurologic cases, with a few exceptions, a definite increase in the reducing power of the system is evident. In approximately 10% of the 92 cases an opportunity was afforded to re-examine the CSF during treatment and at recovery. It was noted, in general, that the initially high neurolytic activity tends to drop to normal levels. The use of enzyme activators revealed high Mg^{++} activation, moderate Ca^{++} activation (Fig. 1). On the other hand we found an incomplete inhibition by *Cl. welchii* antitoxin throughout (Fig. 2). The temperature of incubation is arbitrarily fixed at 37° C. The optimum is above body temperature, around 42° C. (Fig. 3).

Discussion

The phosphorus and sugar-containing organic compounds of the nerve tissue emulsion serve as substrate of this neurolytic enzyme-activity. In our assays the phosphorus and carbohydrate changes sometimes run parallel, sometimes do not. This strongly suggests that not the same enzymes are present in all the cerebrospinal fluids. The presence of phosphomonoesterases, in pathological cerebrospinal fluids was described by Demuth (4), Kaplan (8), Kovacs (13), and Colling and Rossiter (3). The action of nucleases, nucleotidases, and nucleosidase could explain the simultaneous increase of phosphorus and of sugar concentration of the system. The difference in activation by Mg^{++} and Ca^{++} suggests the presence of different enzyme systems, and the incomplete inhibition with *Cl. welchii* antitoxin corroborates this fact (Fig. 1). *Cl. welchii* produces toxin "A" which has lecithinase activity. The antitoxin checks this activity quantitatively (McFarlane (14)). In our assays if there were only lecithinases present the *Cl. welchii* antitoxin would inhibit completely the hydrolysis of lecithin. This is not the case owing to the fact that there are other enzymes also present on which the antitoxin has no effect (Fig. 2).

The accepted opinion is that normal CSF does not possess glycolytic enzymes. In the presence of polymorphonuclear leucocytes, the CSF becomes glycolytic (Chevassut (2)). Table II, Group *D* shows that the glycolysis in our system may conform to this accepted pattern. But there are exceptions: one influenzal meningitis, one meningococcal meningitis, and a case of *E. coli* meningitis with 85,000 polymorphonuclear cells per cu. mm. show a marked increase of reducing substances. In lymphocytic meningitis no change was found. This emphasizes that the relationship between cell count and reducing substance changes is not absolute. There may at least be two different enzyme systems present acting in opposite directions, namely glycolysis,





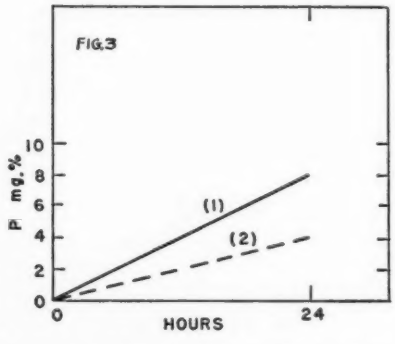


FIG. 1. The effect of Mg and Ca on neurolysis. G.D. Influenzal meningitis. (1) Mg^{++} 0.01% conc. (2) Ca^{++} 0.01% conc. (3) No activation.

FIG. 2. Effect of *Clostridium welchii*. G.D.—Influenzal meningitis. (1) CSF without inhibitor. (2) CSF with 2 cc. *Cl. welchii* antitoxin.

FIG. 3. Effect of temperature on neurolysis. L.P.—Neurosyphilis. (1) CSF without activation, 42° C. (2) CSF without activation, 37° C.

tending to diminish the sugar content, and some diastase-like or phosphorylase-like activity, tending to increase it. In the *E. coli* meningitis case we found a high nucleic acid content in the CSF and high nucleosidase activity (to be published later), which may account for the increase of reducing material. Table II, Group *E* shows in general a definite increase in reducing substances; three spinal fluids show very high values. The cells present in these cerebrospinal fluids are mostly lymphocytes, and there is no detectable glycolysis in the control CSF; thus the carbohydrates set free by neurolytic enzyme action can be detected readily. The same can be said of the remaining specimens of this group, with the exception of one case of subdural haematoma with haemolytic CSF. This group is characterized by a very high neurolytic activity with a considerable increase of reducing substances. These fluids are all poor in cells and have various protein concentrations.

Neurolysis is an enzymic process carried out by simultaneous activity of different enzyme systems. We found no strict relationship between neurolysis and cell count. There is a more strict relationship between the protein concentration and neurolysis. The high protein content, with a few or no cells in the series of very active neurolytic CSF's in our study, suggests that one part of this protein is constituted of enzymes. These enzymes may derive from the nervous tissue as part of the proteins set free by the pathological process. The other main source of the enzymes of the CSF is the blood (Draganesco (5) and Kaplan (9)). We suggest that some extrasystemic cells, such as microorganisms and tumor cells, may play a temporary role in the origin of enzymes of the CSF.

The neurolysis as a complex biocatalytic action may play its part in pathological processes of the nervous system, such as softenings, brain atrophy, demyelination, and repair. The complex system used in the reported assays tends to approach the complexity encountered *in vivo*. We are continuing our studies with simple systems and animal experiments in order to identify the enzymes involved.

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FURTHER STUDIES ON A PITUITARY FACTOR WITH SPECIFIC INFLUENCE ON METABOLISM[1]

By J. B. Derrick and J. B. Collip

Abstract

Pituitary preparations from which known metabolic hormones have been eliminated contain a factor specifically concerned with metabolism. During a six-hour period immediately following the subcutaneous injection of such preparations into fasting rabbits a significant rise in the respiratory quotient (R.Q.) values occurs. The R.Q. values of normally fed rabbits are lowered by similar treatment. These changes are usually, but not necessarily, accompanied by an increase in the metabolic rate. Purified melanophore hormone preparations exert substantially similar effects. Control preparations of liver and muscle tissue have no significant effect on the R.Q. values of fed or fasted rabbits and have a variable effect on the oxygen consumption of these animals.

Introduction

A factor which stimulated an immediate metabolic response was found by Collip and his associates (11, 12) in pituitary extracts from which significant concentrations of known metabolically-active hormones were absent. Injection of the preparations into rabbits, either normal or thyroidectomized, caused an immediate rise in oxygen consumption and in carbon dioxide production, accompanied by a lowering of the respiratory quotient. On the basis of this response and certain biochemical observations (7, 8, 9, 10) it was suggested that the factor might exert its metabolic effects by increasing fat catabolism and sparing the utilization of carbohydrate (12).

A strong melanophore expanding activity paralleled the metabolic effectiveness of the pituitary extracts, and the melanophore hormone and the metabolic factor were shown to have a number of characteristics in common. Upon injection, a highly purified melanophore hormone preparation was also observed to have an immediate stimulating effect on the oxygen consumption of rabbits. The evidence indicated that the two factors were either identical or very closely associated (12). Separation procedures developed by Denstedt and Collip (2) suggested, however, that the melanophore factor and the specific metabolic stimulant were probably not identical.

Teague (17) was unable to show that melanophore hormone preparations consistently produced an increase in the metabolic rate of rats. Pituitary extracts prepared for Teague by the methods of O'Donovan and Collip (12), and similarly prepared control extracts of liver, muscle, and kidney, also gave inconclusive results in the hands of this investigator. A pituitary extract supplied by the Collip group was the only preparation which raised the oxygen consumption of the rats in a consistent manner and to a significant degree. Waring and Landgrebe (18) have reported that melanophore hormone preparations failed to stimulate the metabolic rate of mice.

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Other physiological effects of specific metabolic extracts were also described by Collip and Neufeld (7). These pituitary preparations were found to be antagonistic to the hyperglycemic action of adrenalin and to the hypoglycemic action of insulin and had glycotrophic activity. Similar effects have been attributed to melanophore hormone by other workers (19, 5, 6, 18). This suggests that the presence of this hormone in the extracts could be responsible for their observed effects. Whether these circumstances further implicate the melanophore hormone, or a closely associated factor, in the influence of the specific metabolic extracts on gaseous metabolism is worthy of speculation. Certainly the effects outlined above could not occur to any significant degree without affecting the gaseous phase of metabolism.

The present work is concerned with the influence of pituitary extracts, essentially similar to the earlier specific metabolic factor preparations, and of Intermedin preparations* on the gaseous metabolism of rabbits. It is part of a broader program to determine what changes in the general metabolism the gaseous metabolic effects of the preparations represent.

The experimental results support the earlier evidence that a metabolically active factor is present in the pituitary extracts and in the melanophore hormone preparations. In addition, it has been demonstrated that the factor has an effect on the respiratory quotient which is apparently directly related to the dietary status of the animal. The pituitary extracts and the melanophore hormone preparations have been observed not only to lower the R.Q. in the normal 'fed' rabbit, but also to raise this value significantly when the animal is in a fasting state.

Methods

Methods of Preparation of Extracts

The metabolically active pituitary extracts were prepared as follows:

One hundred grams of frozen beef pituitary glands from which fibrous tissue had been dissected were finely ground in a meat grinder and washed into a flask with 250 ml. of 1% acetic acid. The mixture was heated, with occasional stirring, in a boiling water bath for one hour. A further 2.5 ml. of glacial acetic acid was added half an hour after the processing had commenced. After cooling, the preparation was made up to an 80% alcohol content with absolute ethyl alcohol, and left to stand overnight in the refrigerator. The precipitate was filtered off and the filtrate evaporated at 37° C. to an "almost dry" condition on a vacuum pump. The residue was diluted with approximately 35 ml. of distilled water, adjusted with 5 *N* sodium hydroxide to pH 11.2 and heated in a water bath for 20 min. at a mean temperature of 93° C. After cooling, the pH of the preparation was brought to 7.25 with 3 *N* hydrochloric acid and made up to a 50-ml. volume with distilled water. The preparation was again brought up to an 80% alcohol content, allowed to stand in the cold overnight, filtered, and the filtrate evaporated to the "almost

* *Purified melanophore hormone preparations kindly supplied by Dr. Stehle, Department of Pharmacology, McGill University.*

dry" condition and brought to 50-ml. volume with distilled water. This portion of the procedure was carried out three times and resulted in the production of the extracts referred to as Ip3.

The control extracts were prepared in the same manner. Those made from beef muscle tissue are referred to as MIp3 and those from beef liver as LIp3.

The melanophore hormone preparations were prepared by Dr. Stehle according to his published procedures (13, 14, 15, 16). The "A" product contains 0.67 mgm. per ml. of a melanophore preparation which is 73 times as potent as standard pituitary powder in the frog melanophore dilatation test. With reference to the "B" product, Dr. Stehle states that it is prepared by dissolving a weighed quantity, approximately 15 mgm., of the "A" preparation in about 15 ml. of 0.1 *N* sodium hydroxide. This solution is heated in a boiling water bath for 15 min. and cooled. An amount of 2 *N* hydrochloric acid theoretically equivalent to the sodium hydroxide employed is then added. The preparation is acidified with glacial acetic acid until Congo red indicator turns to a pale blue. It is then diluted so that there is 0.67 mgm. of the preparation per ml., sealed in ampoules in quantities of 1 ml., and the ampoules are boiled for 20 min.

Method of Determination of Metabolic Rate and Respiratory Quotient

The apparatus which was used for the simultaneous determination of the M.R. and R.Q. is a modification of that used in the original open Haldane method (4). The weighings were carried out using a suitably large analytical balance and the calculations were made according to the procedure of Brody (1).

The experimental animals were mature male rabbits. These animals were subjected to several "training runs", the number of runs varying with the temperament of the particular animal. Control measurements were made over a two-hour period immediately preceding subcutaneous injection and the experimental measurements were continued for three consecutive two-hour periods after the injection. When it was required, the experimental animals were fasted for 24 hr. prior to the determination in an effort to bring down the control R.Q. to less than 0.8. Carrots and rolled oats were fed to nonfasting animals on the evening of the day before the determinations in a similar effort to maintain the control R.Q. above 0.9.

Results

The results are based on the values obtained from groups of six rabbits each. Table I offers a summary of the mean differences occurring between the values of the R.Q. and M.R. (oxygen consumption in liters per square meter per 24 hr.) for the control or pre-injection period and those for the three subsequent two-hour, postinjection, observation periods within the various experimental and control groups. The values for the standard error of the mean difference (S.E. mean diff.) and for the probability (*P*) in this table were calculated using the "Student" method (3). Animals with

TABLE I

A SUMMARY OF THE MEAN DIFFERENCES ($\bar{x}$) BETWEEN THE PRE-INJECTION AND POSTINJECTION RESPIRATORY QUOTIENT AND OXYGEN CONSUMPTION VALUES OF RABBITS* AT VARIOUS TIME INTERVALS AFTER SUBCUTANEOUS INJECTION WITH CERTAIN PITUITARY PRODUCTS AND CONTROL PREPARATIONS

Control R.Q.	Subcutaneous injection	$\bar{x}$ diff. R.Q.			$\bar{x}$ diff. O_2 consumption		
		0–2 hr.	2–4 hr.	4–6 hr.	0–2 hr.	2–4 hr.	4–6 hr.
R.Q. > .9	5 ml. 0.9% saline	−.027 ± .008 $P < .02$	.002 ± .008 $P > .05$	−.032 ± .024 $P > .05$	− 6.5 ± 6.9 $P > .05$	−15.3 ± 4.1 $P < .02$	− 5 ± 5.4 $P > .05$
R.Q. > .9	5 ml. MIp3 extract	−.023 ± .013 $P > .05$	−.036 ± .021 $P > .05$	−.038 ± .021 $P > .05$	−2.7 ± 6.0 $P > .05$	− 8.7 ± 7.8 $P > .05$	− 8.2 ± 9.9 $P > .05$
R.Q. > .9	5 ml. LIp3 extract	−.082 ± .024 $P < .02$	−.048 ± .026 $P > .05$	−.067 ± .019 $P < .02$	7.2 ± 6.7 $P > .05$	− 5.0 ± 5.2 $P > .05$	− 1.8 ± 3.6 $P > .05$
R.Q. > .9	0.67 mgm. Intermedin A in 5 ml. saline	−.052 ± .019 $P < .05$	−.108 ± .009 $P < .01$	−.102 ± .029 $P < .02$	27.2 < 5.2 $P \pm .01$	30.5 ± 8.7 $P < .05$	11.3 ± 8.7 $P > .05$
R.Q. > .9	0.67 mgm. Intermedin B in 5 ml. saline	−.087 ± .021 $P < .02$	−.108 ± .017 $P < .01$	−.105 ± .012 $P < .01$	14.5 ± 3.5 $P < .01$	12.7 ± 4.1 $P < .05$	9.8 ± 6.2 $P > .05$
R.Q. > .9	5 ml. Ip3 extract	−.013 ± .008 $P > .05$	−.100 ± .010 $P < .01$	−.107 ± .012 $P < .01$	11.0 ± 4.6 $P = .05$	12.7 ± 2.2 $P = .05$	14.3 ± 2.6 $P < .05$
R.Q. < .8	5 ml. 0.9% saline	−.016 ± .009 $P > .05$	−.005 ± .012 $P > .05$	.000 ± .014 $P > .05$	.33 ± 4.9 $P > .05$	−5.03 ± 3.3 $P > .05$	− 3.3 ± 4.1 $P > .05$
R.Q. < .8	5 ml. MIp3 extract	.003 ± .002 $P > .05$	−.012 ± .011 $P > .05$	−.007 ± .022 $P > .05$	15.2 ± 3.3 $P < .01$	13.3 ± 4.8 $P < .05$	18.5 ± 7.5 $P = .05$
R.Q. < .8	5 ml. LIp3 extract	.000 ± .008 $P > .05$	−.015 ± .006 $P > .05$	−.015 ± .007 $P > .05$	8.3 ± 5.9 $P > .05$	3.8 ± 6.2 $P > .05$	3.3 ± 9.1 $P > .05$
R.Q. < .8	0.67 mgm. Intermedin A in 5 ml. saline	.033 ± .017 $P > .05$	−.007 ± .015 $P > .05$	−.022 ± .021 $P > .05$	34.2 ± 6.8 $P < .01$	36.8 ± 6.0 $P < .01$	26.7 ± 7.9 $P < .02$
R.Q. < .8	0.67 mgm. Intermedin B in 5 ml. saline	.107 ± .009 $P < .01$	.003 ± .007 $P > .05$	−.013 ± .013 $P > .05$	6.0 ± 5.6 $P > .05$	39.8 ± 8.4 $P < .01$	41.3 ± 10.0 $P < .02$
R.Q. < .8	5 ml. Ip3 extract	.102 ± .017 $P < .01$	.075 ± .022 $P < .02$	−.000 ± .012 $P > .05$	10.8 ± 14.5 $P > .05$	18.0 ± 6.0 $P < .05$	21.7 ± 7.8 $P < .05$

* *Representing results of determinations on six animals for each preparation.*

initial R.Q. values of less than 0.8 have been fasted for 24 hr. prior to the metabolism determination and those with R.Q. values greater than 0.9 have been normally fed.

From the data in Table I it may be observed that there is a marked tendency for the mean difference between the pre-injection and the postinjection R.Q. values to be positive when fasted rabbits (i.e. pre-injection R.Q., less than 0.8) are injected with the pituitary extract Ip3, or with Dr. Stehle's melanophore preparation Intermedin B.

Statistical evaluation based on a comparison of the R.Q. responses of the various experimental and control fasted groups similarly demonstrates this trend. A significant difference is indicated when the ability of the Ip3 extract to raise the R.Q. in a group of fasted animals is compared statistically with that of the control extract MIp3. For the first two-hour period after injection P is less than 0.1 and for the second, less than .02. No difference between the two extracts is apparent for the third two-hour postinjection period. A comparison of Ip3 with control extract LIp3 also discloses a significant difference in the ability of these two extracts to raise the R.Q. of fasted groups, since P is less than .01 for both the first and second two-hour postinjection periods. Again, no significant change is apparent for the third period after injection.

Comparison of the mean difference in R.Q. occurring between fasted groups injected with Intermedin A in physiological saline, or with an equivalent quantity of saline, shows that the former preparation raises the R.Q. significantly higher than does the control preparation for the first two-hour period after injection (P less than .05). Similarly, Intermedin B gives a significant increase in R.Q. value as compared to the saline-injected control group during the first postinjection period (P less than .01) with no significant difference apparent for the remainder of the observational periods.

The data in Table I also show that when fed rabbits (R.Q. greater than 0.9) are injected with the Ip3 pituitary extract, Intermedin A, or Intermedin B, there is a general trend toward a lowering of the R.Q. and the mean differences observed between the pre-injection and postinjection values within these groups are mainly negative.

Statistical comparison of the Ip3 injected group with the group receiving control extract MIp3 shows that the R.Q. is significantly lowered in the former group for the second and third postinjection observation periods ($P = .02$). Less clearly defined is the comparison of the activity of Ip3 and LIp3 extracts. Here, the control extract is found to lower the R.Q. of a fed group of animals during the first postinjection period significantly more than does the Ip3 extract. No difference between the two extracts is apparent in a comparison of the groups for the second period after injection and for the third period there is a barely significant difference in favor of the Ip3 extract (P is slightly greater than .05).

Fed groups receiving Intermedin A in physiological saline or an equivalent quantity of saline show the former preparation to be significantly active in

lowering the R.Q. during the second and third postinjection periods since P is respectively less than .02 and less than .01 for these two periods. The fed group receiving Intermedin B shows a significant lowering of the R.Q. in all three postinjection periods when compared to the control, saline injected group. For the first period, P is less than .05; for the second period, it is less than .01, and for the final observation it is again less than .05.

Fig. 1 is a graphic presentation of the mean difference in the R.Q. values of the various groups. The abscissa of the figure represents the mean initial R.Q.

An increase in the M.R. values of the experimental groups is generally apparent throughout the work (Table I). The data reveal, however, that there is evidence of a lack of parallelism between the R.Q. and the M.R. changes. Significant changes in the R.Q. occur, at times, unaccompanied by significant changes in oxygen consumption, and vice versa. In several instances, more notably in the fasted animals, there is a tendency for the changes in oxygen consumption to lag behind the changes in the R.Q. and the degrees of change in the two values sometimes fail to correlate well.

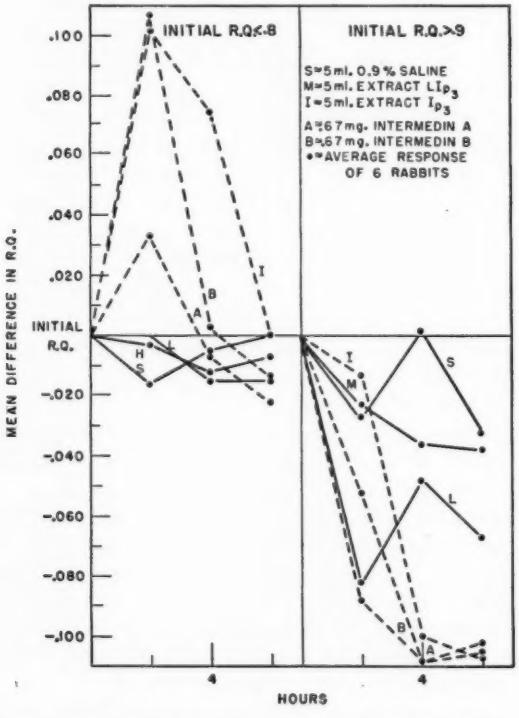

FIG. 1. The mean differences in the respiratory quotients of rabbits at two, four, and six hours after subcutaneous injection.

These observations are less easily seen in the summarized data of Table I than in the data on individual animals, not presented here because of space considerations.

Fig. 2 is a graphic presentation of the mean differences in the M.R. values of the various groups. In this figure the abscissa represents the mean initial M.R.

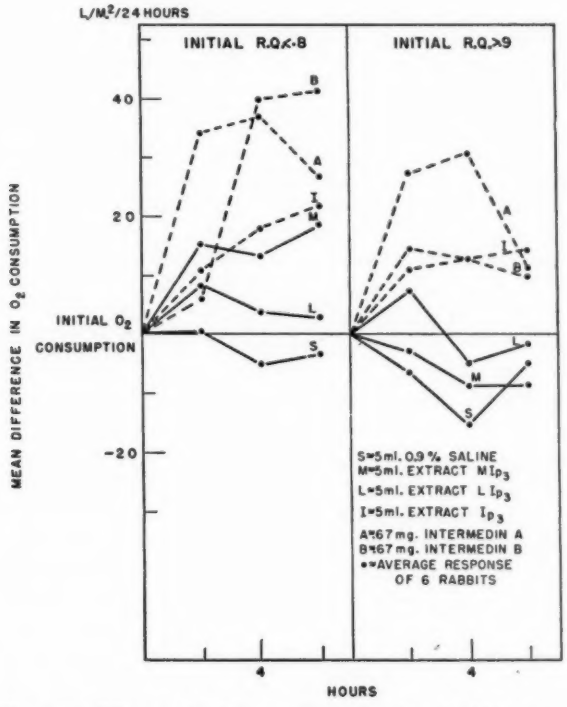

FIG. 2. The mean differences in the oxygen consumption of rabbits at two, four, and six hours after subcutaneous injection.

Discussion

The experimental results of the present work are in general agreement with the previous observations of Collip and his associates on the existence of a factor specifically concerned with metabolism and present in pituitary preparations from which significant concentrations of the known metabolically active hormones are absent. The significant increase in oxygen consumption and the immediacy with which this metabolic response occurs, following the subcutaneous injection of these preparations, are substantiated by the data presented above.

In the present work greater emphasis has been placed on the effects of the specific metabolic factor on the respiratory quotient. It has been found that

the various pituitary preparations are capable not only of lowering the R.Q., as reported in the earlier investigations, but also of raising this value, depending on the dietary status of the animal. Further, it has been demonstrated that an effect on gaseous metabolism which may not be apparent in the M.R. values may be observed in the R.Q. While the converse could also be true, preparations which stimulated an increase in oxygen consumption without a concomitant change in the R.Q. have not been considered metabolically-active in the present work since the unpredictable nervous reactions of the rabbits to experimental procedure complicate the assessment of this response. It is possible that dependence on the changes occurring in the metabolic rate alone may be responsible, at least in part, for the failure of other investigators to demonstrate the specific metabolic effect.

A brief series of determinations of the alkali reserve of the experimental rabbits has not indicated that the changes in the R.Q. values are due to changes in the carbon dioxide combining power of the blood. All the values were within the accepted normal limits. Whether or not fluctuations within the normal limits could influence the R.Q. to the extent observed in these investigations has not been determined at the present time. It is possible that expired carbon dioxide from preformed bicarbonate might be responsible for the positive order of the change observed in the R.Q. values of the fasted experimental rabbits. Similar reasoning cannot be applied to the fed animals, however, in view of the negative order of the R.Q. changes in this case.

Consideration of the tendency for the R.Q. of fasted or fed rabbits to be forced upward or downward respectively, in response to the subcutaneous injection of preparations containing the specific metabolic factor, results in the conclusion that while this factor may still be referred to as being of a stimulatory nature it could equally well be referred to as being of a regulatory nature. The manner in which this regulation of the metabolism is effected, however, is difficult to interpret at the present time.

On the basis of the evidence of both past and present work, it may still be considered that the factor is in some way concerned with the catabolism of fat. The newer evidence with the regard to the R.Q. response suggests, however, that its effects on carbohydrate metabolism may not necessarily be concerned with the sparing of carbohydrate alone. Rather they are indicated to be more of a regulatory nature and may quite possibly be involved, either as the result of direct or indirect influence by the factor, with an increased efficiency of utilization of the products of fat breakdown. Such a mechanism would be consistent with the presently observed values for the R.Q.

It is not thought that the fatty portion of the pituitary preparation is responsible for the effects on metabolism. Theoretically it might be expected to cause lowering of the R.Q. values but it is unlikely that it would be responsible for raising them. Injection of the fatty fraction into rabbits has shown that it is very much less active metabolically than is the whole extract. Further, this fraction is slowly and poorly absorbed and, therefore, would be unlikely to elicit an immediate response.

Summary

The R.Q. values for rabbits in the fasting state are significantly raised during a six-hour period immediately following a subcutaneous injection of a specific metabolic factor pituitary extract. Conversely, the R.Q. values of fed rabbits are significantly lowered under similar conditions. An increase in oxygen consumption is generally apparent, although it does not necessarily accompany these effects and, indeed, may occur in their absence.

Control preparations of liver and muscle tissue have no significant effect on the R.Q. values of fed or fasted rabbits and have a variable effect on the oxygen consumption of these animals.

Purified melanophore hormone (Intermedin) preparations have been observed to exert effects substantially similar to those of the specific metabolic factor pituitary preparations on the gaseous metabolism of rabbits.

On the basis of the above evidence it appears probable that the factor responsible for the effects on gaseous metabolism is common to both preparations.

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